ISTITUTO LOMBARDO - ACCADEMIA DI SCIENZE E LETTERE

DEGRADATION OF DEOXYRIBONUCLEIC ACID BY ACID DEOXYRIBONUCLEASE

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Treatment of kinetic data

Schumaker, Richards and Schachman [7] have given a general theory for the degradation of multi-stranded polymers, of any initial distribution of molecular weights. They have shown that for the initial stage of degradation the following equation holds:

$$\log(1 - R) = n \log p + const \qquad (1)$$

Here $R = M_t/M_o$; (M_t and M_o are the molecular weights at time t and at time zero, respectively); n is the number of strands and p is the probability that any given bond is broken. Equation (1) shows that a plot of $\log(1 - R)$ vs. $\log p$ will have a slope equal to n. A more convenient plot is obtained by replacing, as proposed by Cavalieri and Rosenberg [8], $(1 - R)$ by $(1 - R)/R$ in equation (1). Then, assuming that $p = k t$, (k being a proportionality constant) equation (1) becomes:

$$\log(1 - R)/R = n \log t + const \qquad (2)$$

The use of equation (2) has the advantage that $\log(1-R)/R$ is a linear function of $\log t$ over a wider range of R values. For the particular case where $n = 1$, equation (2) becomes

$$\frac{1}{M_t} = k t + const \qquad (3)$$

which is exactly what the statistical theory of random degradation of linear polymers predicts [9].

Methods and Materials

The enzymatic degradation of DNA has been followed by digesting DNA solutions directly in the cell of a Wippler and Scheibling light-scattering apparatus [10] and measuring at different time intervals the intensities of the light scattered at six angles comprised between 30° and 90°. The amounts of enzymes added were such as to obtain a change of R from 1.0 to 0.7 in about 2 hours. The validity of the light-scattering technique in assessing

Results

As a term of comparison, let us consider first the results we obtained when digesting DNA with pancreatic (neutral) DNase. The kinetics of degradation of DNA by pancreatic DNase has been shown by Thomas [13] and by Schumaker *et al.* [7] to be
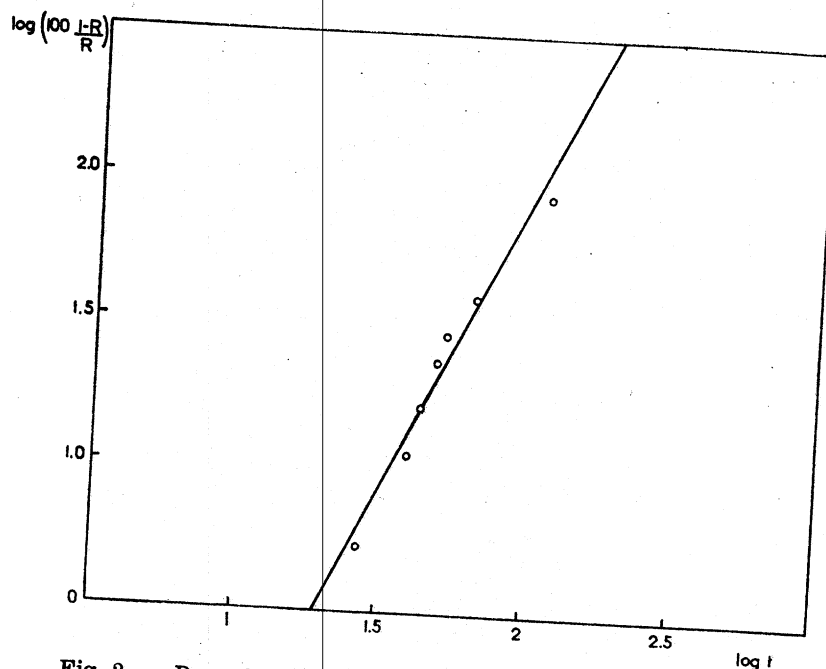

Fig. 2. — Degradation of native DNA by pancreatic DNase.

a « double hit » process. The enzyme attacks at random phosphodiester bonds on both DNA strands; a decrease in molecular weight takes place only when opposite breaks occur; this happens after a lag time during which random breaks accumulate on each parent molecule.

Accordingly, in the degradation by pancreatic DNase we find a definite lag time and when the molecular weight starts to decrease, (1/M) increases quadratically with time. In the double logarithmic plot we find $n = 2$ (figures 1 and 2).

However, the « single hit » degradation is not the only active process during the digestion, as is shown by the following lines of evidence:

a) Titration data obtained by Dr. Richards in our laboratory show that phosphodiester bond splitting is linear with time down to extremely low molecular weights (this finding justifies the replacement of p with t in equation (2)). For a given decrease
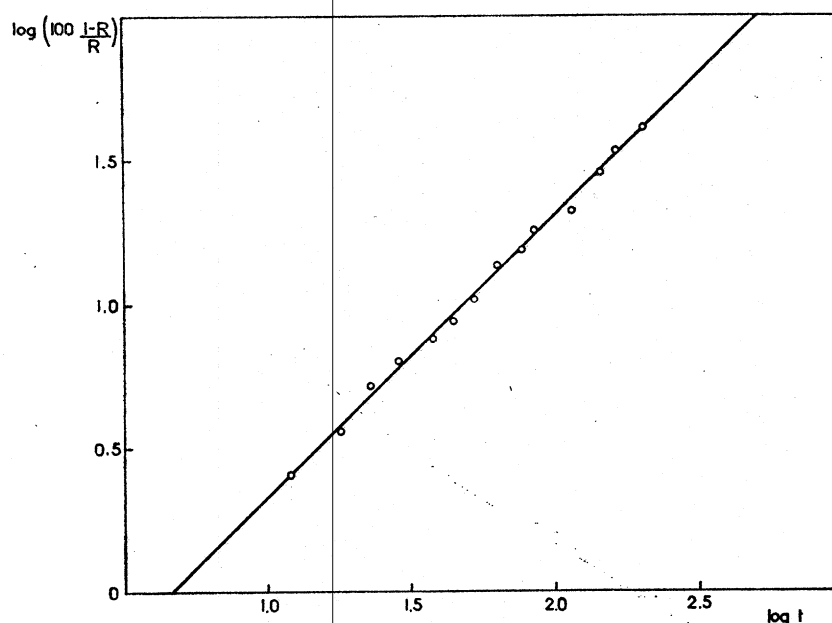

Fig. 4. — Degradation of native DNA by acid DNase.

in molecular weight (down to 0.5×10^6) the number of bonds broken is larger than expected for a pure « single hit » kinetics, but much smaller than for a « double hit » kinetics. Our data show that we need at most 10-20 breaks per parent molecule of 6.10^6 molecular weight to reduce it by a factor of two, whereas 200 breaks are needed in a « double hit » degradation [13] and about 3 in a pure « single hit » degradation in order to obtain the same result.

d) A fourth line of evidence is provided by the degradation of heat denatured DNA.
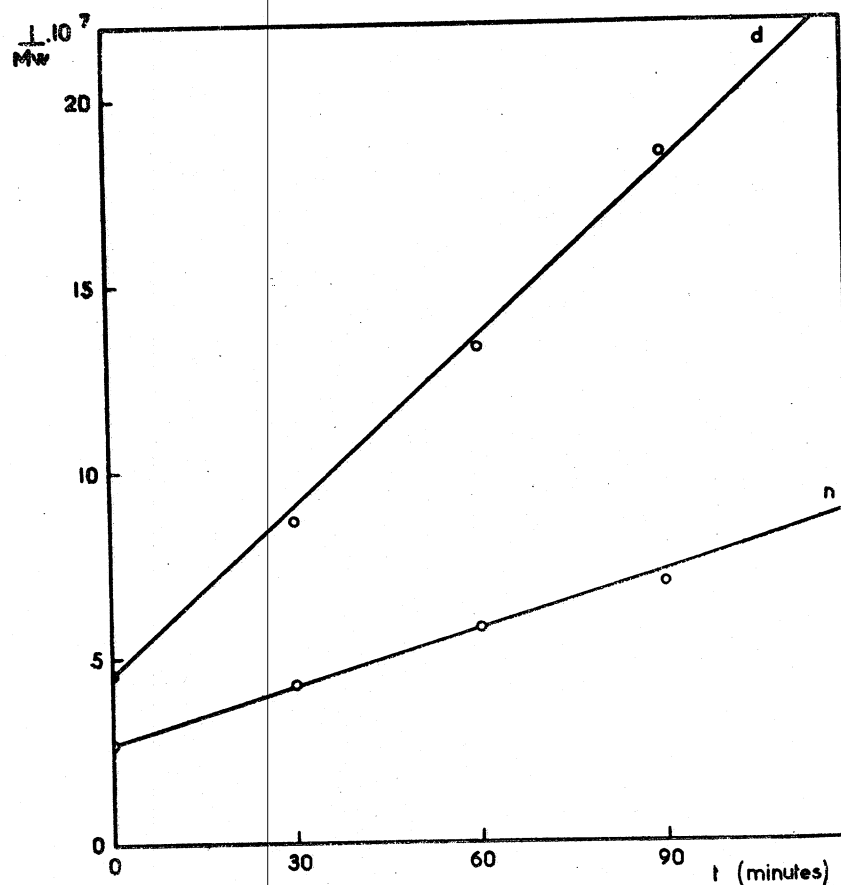

Fig. 6 — Effect of heat denaturation upon the molecular weight of DNA samples obtained at different times of digestion with acid DNase. Upper line: denatured samples; lower line: native samples.

2) Denatured DNA.

DNA samples, heated for 10 minutes at 100°C, and fast cooled, showed, upon digestion with acid DNase, a « double hit » kinetics (figures 7 and 8). This result is in agreement with similar results obtained by Cavalieri *et al.* [8]. Apparently, the sites susceptible to the « single hit » action do not exist any more in heat

denatured DNA. The degradation, as measured by acid-soluble nucleotide formation, is slower, by a factor of two, for heat denatured DNA than for native DNA (figure 9).

On the other hand, DNA denatured by electrolyte dilution at 20°, once brought back to 0.15 ionic strength, behaves like native DNA towards acid DNase.
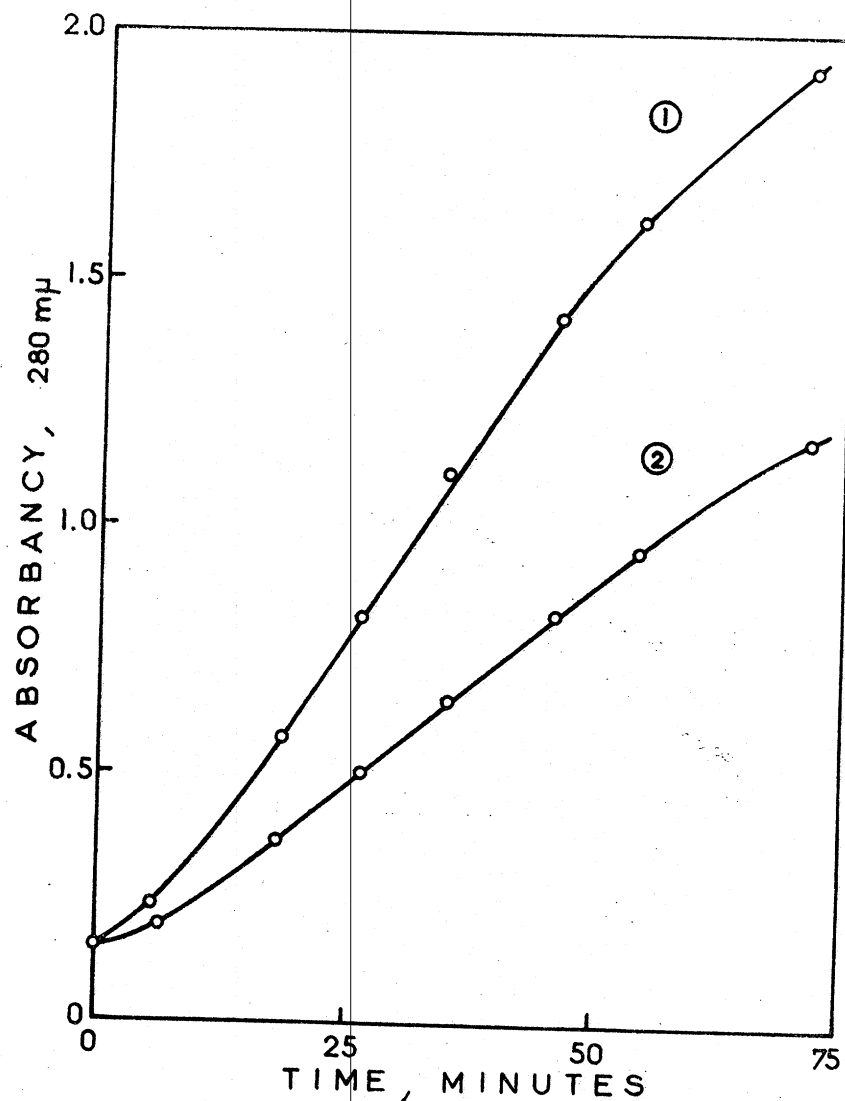
Fig. 9. — Acid-soluble nucleotide formation plotted *vs* digestion time by acid DNase. Upper curve: native DNA; lower line: heat-denatured DNA.

1) a « single hit » kinetics in which (if DNA molecules are made up of two uninterrupted filaments), both strands are simultaneously split at the same site;

2) a « double hit » kinetics in which random breaks occur on one strand at a time as in the case of pancreatic DNase. This second type of kinetics is not evident in the early stage of degra-
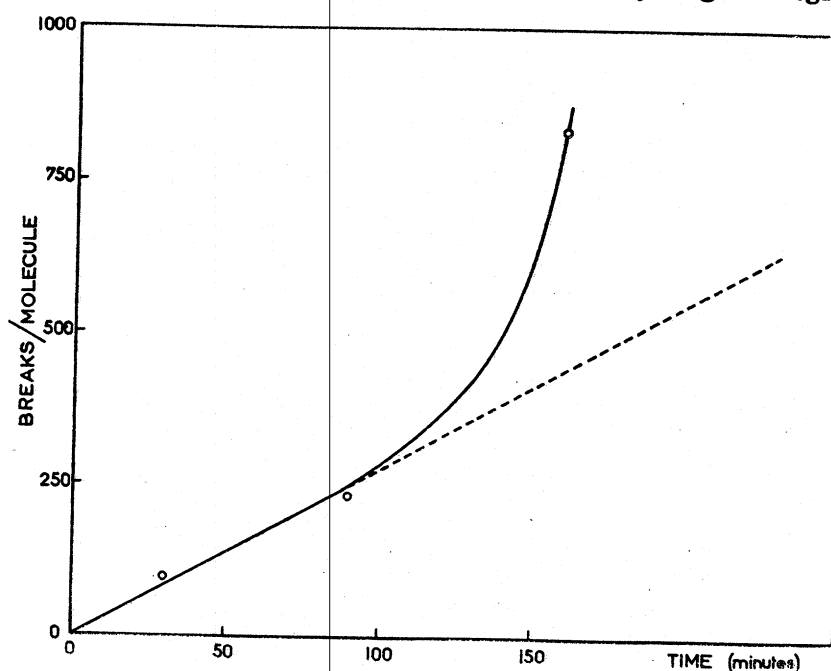

Fig. 11. — Degradation of T5 bacteriophage DNA by acid DNase (data calculated from results presented in ref. 12).

dation because of its own lag time, and only becomes apparent in a later stage.

Interestingly enough, these conclusions were confirmed last year in Baltimore by Mac Hattie, Bernardi and Thomas [12] using a completely different experimental approach. DNA from T5 bacteriophage was digested with acid DNase and the fragment size distribution at three time intervals was studied by electron microscopy. When plotted against the digestion time, the number of scissions showed, after an initial linear part, an upward curvature; therefore the plot (figure 11) was very similar to that shown

3) Sheared DNA.

Sheared DNA shows a behaviour intermediate between those of native and heat denatured DNA. Values of n ranging from 1.3 to 1.5 are generally found. The behaviour is therefore the one ex-
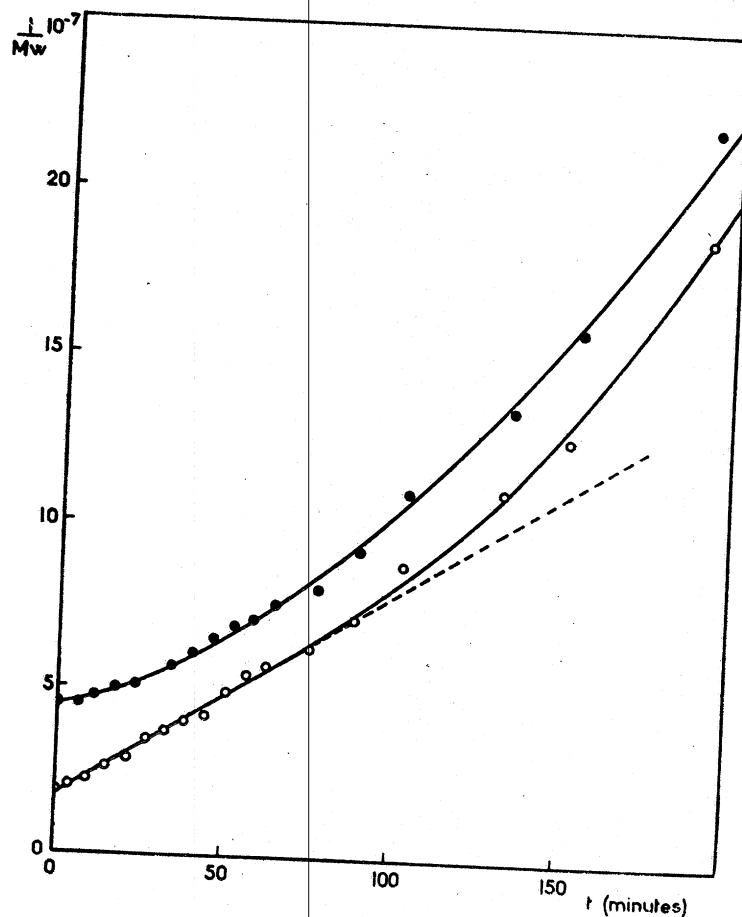

Fig. 10. — Degradation of native intact DNA (lower curve) and of sheared DNA (upper curve) by acid DNase.

pected for a DNA having lost a part of its sites susceptible to the single hit action (figure 10).

On the basis of the above data we conclude that degradation by acid DNase takes place according to two different mecanisms:

We would like to discuss briefly here only some of the points raised by the results reported above.

Except for a few « nicks » caused by the « double hit » action, the DNA fragments obtained by acid DNase digestion (at least down to a molecular weight of 0.5×10^6), have the structure of native DNA. They show no hyperchromicity, no acid-soluble oligonucleotides formation (both of these phenomena showing a lag time) (figure 12), a melting curve which does not differ from that
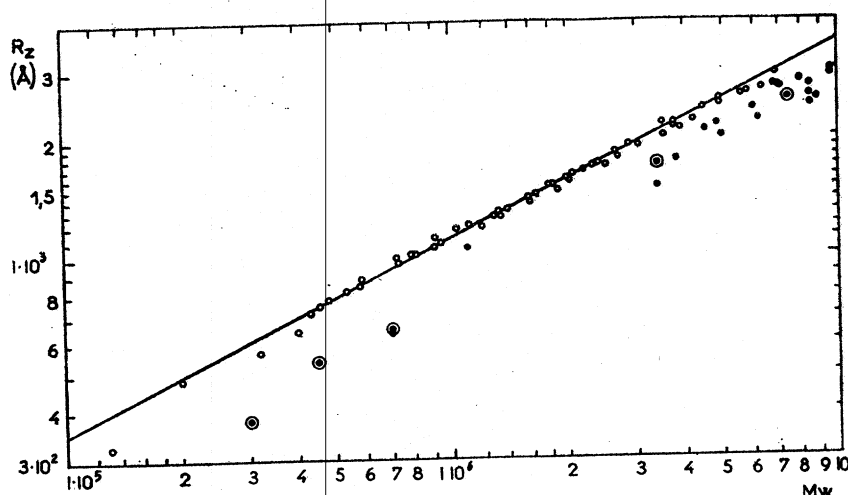

Fig. 13. — Variation of radius of gyration with molecular weight for DNA samples digested with acid DNase (circles). Data of Lett and Stacey (14) are shown as points; data of Doty et al. (15) as circled points.

of undigested DNA, a particle width close to 20 Å, and a molecular weight per unit length of 200 daltons/A. Therefore DNA fragmented by acid DNase should be a useful material for measurement of physical properties which are difficult to assess when using the very viscous solutions of high molecular weight DNA and, in addition, a host of physico-chemical investigations could also be profitably performed on this material.

On the other hand, the fact that by acid DNase digestion double stranded fragments of DNA of any molecular weight between 6×10^6 and 0.5×10^6 may be obtained, allowed us to esta-

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in figure 5. Electron microscopy also showed that the fragment size distribution was random, and that the fragments had the same width as native DNA.
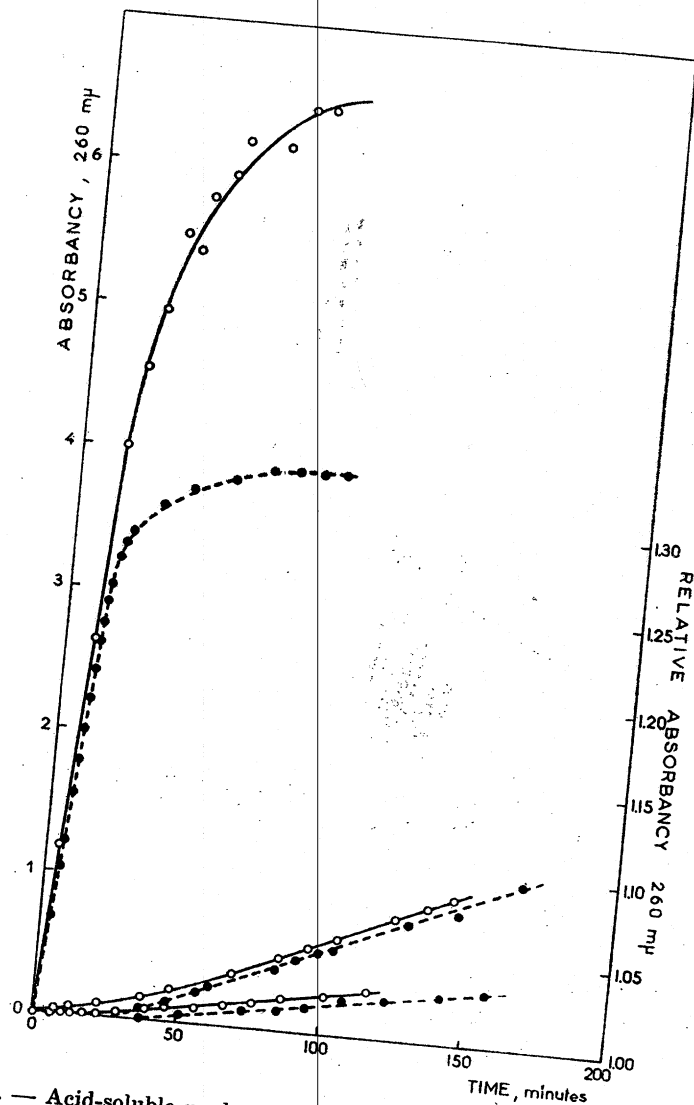

Fig. 12. — Acid-soluble nucleotide formation (circles; left-hand ordinate) and hyperchromic effect (points; right-hand ordinate) plotted *vs* digestion time of DNA by acid DNase. The three sets of curves refer to three different enzyme concentrations.

thought to cause double breaks on the DNA molecules):

$$S_{20,w} = 0.063 \times M^{0.37} \qquad (7)$$

$$[\eta] = 1.45 \times 10^{-4} M^{1.12} \qquad (8)$$

$$R = 0.27 \times M^{0.58} \qquad (9)$$

As far as the mechanism of the enzymatic degradation is concerned, much work remains to be done before we can reach a complete understanding of the processes involved.

At the present moment we think that two working hypotheses deserve some consideration:

1) DNA molecules are formed by sub-units connected by « linkers », for instance, phosphoserine residues as suggested by Welsh [16] and, more recently, by Bendich and Rosenkranz [17]. Acid DNase would be able to split phosphodiester bonds involving serine, thus providing a « single hit » action; the « double hit » action would take place, instead, on the normal internucleotidic links. Since it has been shown very recently in our laboratory [18] that pure acid DNase also has a phosphodiesterase activity and is able to split even synthetic substrates like Ca [bis(p-nitro-phenyl-phosphate]$_2$, an activity on an hypothetical nucleotidyl-serine linkage cannot be ruled out a priori. The main trouble with this hypothesis is therefore the lack of a definite evidence for the existence of serine « linkers ».

2) Acid DNase is a dimeric protein molecule and its mechanism of action is formally analogous to that of difunctional alkylating agents. Alternatively the enzyme might have two active sites.

This second hypothesis does not involve any arbitrary assumption of non-nucleotidic material interpolated along the DNA duplex. It is based upon a chemical model, namely the degradation of DNA by alkylating agents, as studied by Lawley and Brookes [19]. We will briefly recall the main points of this work. Under a given set of experimental conditions, alkylation has been shown to occur at N_7 of guanine, monofunctional agents yielding 7-alkylguanines, and difunctional agents yielding, in addition, di(guaninyl) derivatives. This latter event has been shown to occur only when two guanines find themselves on opposite strands

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Discussion following Dr. BERNARDI's paper.

Dr. Dulbecco: I want to recall one finding we observed recently while working with the polyoma virus. We studied the kinetics of the change of the ring into the rod by DNAase. One cut produces a rod out of the ring. To our great surprise we found that it was a first order kinetic. This is DNAase I at pH 8.1 in sodium chloride 0.1 M. This shows that there are special factors involvd in determining the kinetics. The striking fact in this particular system is that, although the kinetics of the opening of the ring is single hit, the kinetics of further breaks is 2 hit. It is really the configuration of the molecule which, in this case, determines the kinetics of DNAase digestion as single hit rather than 2 hit. We have, furthermore, evidence that, while this first break is produced, there are no single chain braks. This was obtained by sedimenting the material at pH 12.5. This result is rather surprising and I do not really know how to explain it.

Dr. Bernardi: I do not know if I really caught your point. You have a ring DNA, you treat this ring DNA with pancreatic DNAase and you get a single hit action. Which way was the single hit kinetics determined?

Dr. Dulbecco: By determining the proportion of the ring surviving as a function of time, in the regular kinetic way.

Dr. Bernardi: I do not know if it is enough to follow the two peaks in the ultracentrifuge as a function of digestion. In order to study this kinetics you would really need to work between R values of 1 and 0.5. R = 0.5 corresponds to one hit per molecule, roughly speaking. So I really do not know if you can do it by sedimentation.

Dr. Dulbecco: You have every reason to believe my data.

Dr. Bernardi: I believe your data. It is the interpretation which I doubt.

as in figure 15. Alkylated DNA decomposes with loss of the alkylated guanines and subsequently the corresponding phosphodiester bonds are hydrolyzed. Therefore the net result is that alkylation by monofunctional agents gives rise to a random degradation of DNA of the type obtainable with pancreatic DNase. Alkylation
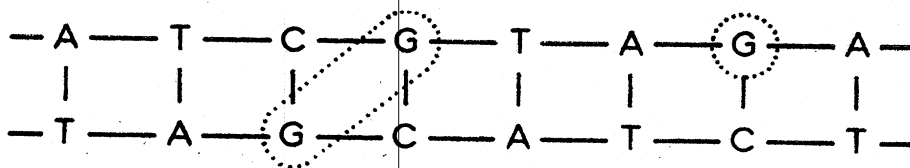

Fig. 15. — Diagrammatic representation of the possible scissions of a DNA chain operated by a difunctional alkylating agent (modified from ref. 19).

by difunctional agents causes a degradation which involves both « single hit » and « double hit » kinetics, simulating therefore the action of acid DNase. (It should be pointed out however that the above schemes are rather idealized pictures because, for example, the « double hit » kinetics expected with monofunctional agents was not found experimentally [20].

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Dr. Dulbecco: I think the data are all right and you can see them published, but the fact is that if you plot the log of the surviving proportion of what remains as a ring vrs. the time of action of DNAase, the curve is not limited to 0.5, because one can follow the conversion of the rings to rods until the band of the rings completely disappears. As it receives one hit this material passes into the other band and therefore the measurements are very accurate. But the material which has been transformed into S, or the native rods, show the classical kinetics. So, it is the same DNA, with the same molecular weight, the only difference is in the configuration; in the case of the ring, one hit breaks both chains; in the case of the rod we have the usual kinetics.

Dr. Bernardi: I think these data are very puzzling. The ring shape is by itself not a factor, because Sinsheimer has shown that the ØX DNA, which is ring shaped and single stranded, behaves as expected using pancreatic DNAase.

Dr. Thomas: I would like to continue this argument. If I understand your suggestion correctly, Dr. Dulbecco, the picture in your mind is that the pancreatic DNAase is breaking the phosphodiester linkages. Therefore, if you were to say that it is single hit, this supposes that there is a single chain region in the polyoma circle, and breaks are directed preferentially towards this region where it is effective immediately. I believe you brought up this question because you are suggesting to Dr. Bernardi that there are single chain regions in the DNA molecule which may, in fact, be nicked, the nick resulting in complete cleavage. Am I a mind reader?

Dr. Dulbecco: May I just answer the facts. Actually, there is very good evidence that there are no single strand fragments in this ring; it is entirely double strand.

Dr. Thomas: But what is the confidence for that statement; are there only 2 or 3 open splices?

Dr. Dulbecco: The rate at which these rings are broken is of the same order of which single strands are broken after the first break is made. If there were a single strand area it would be a very large one and we can exclude this in a most catagoric way. The only thing we can not exclude would be something which is less that 1% of the total lengths, but then the kinetics, the rate, would be much smaller.

Dr. Thomas: May I ask one more question? In one of your slides, Dr. Bernardi, you had the change of molecular weight of the duplex and of the single chain form, and the slopes of these two lines were not parallel, but widely different as a function of time. This presumably would allow you to calculate the number of "nicks per chop" and I wanted to know how that agreed with the titration data.

Dr. Bernardi: From the data shown in Fig. 6, one would estimate less "nicks per chop" than from titration.